BI Flow Facility: Cytek Aurora Training

- Go through the fluidics of the machine and how to change them.

  Explain that if the fluidics need changing, they will get a warning message on the screen stating that they have 5 minutes of sheath fluid left (they should be aware of this if they are leaving the machine to run a plate). The symbols next to waste and sheath will turn from green to yellow if this is the case.

- Explain how to turn on the machine and explain that they need to use the Acquisition tab to start a tube of water running for at least 30 minutes before running QC.

  They will need to wait and make sure the cytometer is connected and the sheath and waste levels are fine before starting to run a tube. They can use the default experiment to run this tube-the default experiment is set up so that there is a histogram for every channel and a spectral plot.

- Go through the training slides with them to introduce them to the Aurora and spectral analysis.

  (See separate PowerPoint presentation with notes.)

- Set them up with an account on the SpectroFlo software. Show them how to load a tube.

  Make them aware that when they unload a tube, they should be careful not to catch the sample line which stays down after cleaning. Explain that in-between tubes the sample line gets taken up and there is a SIT flush before they can run another sample.

- Explain the QC to them. The QC measures the CV of the peak in each channel using single peak beads and calculates the changes in gain required to keep the peak in the same place each time.

  Explain that the beads need vortexing and 1 drop should be added to ~300µl PBS. There must be at least 100 events per second going through in order for the QC to pass. They will be able to see when the last QC was run-the QC should be run every day. If the QC fails then they should first try making up new beads and running it again. They can also run two fluidics shutdowns (the first one using 30% Contrad followed by water, the second one using bleach followed by water). If the QC still doesn’t pass they should inform the Flow Team who will advise them on if they can go ahead with their experiment.

- Go through the Acquisition module with them and show them how to set up an experiment.

  Explain that they can and should save a template if they want to re-use an old experiment (just like they do on the other analysers). They can also import old experiments or duplicate without data but they should be aware that (particularly as the machine gets busier) these experiments will be deleted from the database on a monthly basis unless they request for one to be retained.

- Go through the Fluorescent Tags tab. First they should create the reference controls by selecting the fluorochromes they have in their panel.

  They should make sure all fluorochromes are entered here as these will determine the reference controls. They should then select their negative controls. Remind them that these should be matched (beads for bead controls, cells for cells). Remind them that they always need a negative control to take any background auto-fluorescence into consideration. They can have more than 1 negative control.

- Go through the Groups, Markers, Keywords, and Acquisition tabs.

  They can add labels for each tag here and keywords if they like. They should link their controls and samples to acquisition worksheets. Their reference controls should be linked to a raw worksheet and their samples should be linked to an unmixed worksheet. They can create specimens (groups) and tubes here. Groups for samples can be created by selecting the Group icon and tubes can be added to the group using the + Tube icon. In the Acquisition tab they can define a stopping rule based on events, time, or volume. Select the top level to apply the setting to all the tubes/wells in that group. Remind them to save their experiment.

- Go through how to run and record a tube (Acquisition Control settings).

  To run samples they should load a tube on the SIT and then push Play. When they run samples they should make sure that the green arrow is next to the tube that they want to record. They should wait a couple of seconds before recording so as not to record when the flow is turbulent. They can also change the flow rate here (comparable to the Fortessa in tube mode) and perform extra SIT flushes if needed. Remind them that they should run cleaning tubes or perform a fluidics shutdown (Bleach followed by water) if they are running a lot of tubes.

- Go through how to check if the assay settings (gains) are correct (Instrument Control settings).

  The recommended way of checking assay settings is to put a fully stained sample on and check the spectral plot to ensure that all channels are on scale. Get them to run a fully stained sample to check the settings. Remind them that the Cytek Assay Settings should be appropriate for most panels. They may find that certain antibodies particularly viability dyes need to be titrated down for the Aurora. Remind them that although they can change the assay settings they need to make sure that they are not changing the spectral profile of any of their fluorochromes by doing so. It is better to change the gains across the whole laser rather than a single channel (particularly if they have fluorochromes that are spectrally similar to each other). Show them how to change and save (‘Save As’) the assay settings. They should also use their fully stained to set the scatter voltages. Remind them that the scatter comes from the blue and violet lasers and will be much lower than they are probably expecting compared to the Fortessa. If they are running very large cells they can reduce the FSC area scaling factor.

- Get them to run and record their reference controls. Once they have done this go through the process of unmixing in the software with them.

  Between 100 and 2000 events for each of the negative and positive population is the minimum needed for accurate unmixing. They should click the unmixing icon; adjust the gates in scatter and on the histograms and spectral plot. They can use the Ctrl button when moving a gate to apply it to all the controls. They should make sure that the histogram plot is displaying the correct channel. They should take the time to check this - it doesn't always display the correct one. For example dyes in the yellow green such as PE and PE-tandems come up with a peak in blue laser channels as when the software was released it was only designed for a 3 laser version of the machine. They can use the Cytek guide to check the peak channel for each fluorochrome they are using (show them where this is on the computer). They should make sure to take the brightest part of the positive peak and ensure that the population they are taking for the positive produces a neat spectrum that matches the example one given by Cytek for each fluorochrome.

  They should then click on Live Unmixing or Unmix, Save and Open if they want to do post-acquisition unmixing (this saves the raw data files in the original experiment and opens a new analysis experiment where unmixed fcs files are saved). If one of the controls needs it they can re-acquire or append to it then unmix again. They should run unmixing on the same day as they run their experimental samples either live or post-acquisition. If they run controls and samples as tubes rather than reference controls then they can use the Extra Tools module to select the correct ones for post-acquisition unmixing.

- Explain briefly the process of unmixing.

  The principal of unmixing is the same as compensation but is mathematically different. Instead of calculating the amount of spillover into each non-primary detector the Aurora looks at the spectra across the whole range so tries to pull out the pattern from the fully stained sample. It uses an ordinary least squares regression calculation to get a best fit between what is observed in the fully stained sample compared to the single colour controls. This is why accurate controls are important, because the closer the single colour spectra is to the fully stained spectra, the easier it will be to pull out-a small error makes the best fit closer (so unmixing is more accurate). This is also why you can pull out auto-fluorescence as a parameter and why fluorochromes that are spectrally similar are more difficult to unmix.

- Show them how to create an unmixed worksheet – how to make plots and gates.

  Before running samples they may want to create an unmixed worksheet to display their data. Get them to create a few plots, gates, and bring up a population hierarchy. Show them that they can bring up a statistics table if they want one (click on the Statistics icon in the toolbar and then click on the worksheet – there may be a small delay before it shows up). This is useful if they want to know the volumetric count in a sample. This table can be exported as a csv. Show them that to make changes to plots they should right click on a plot and select Plot Properties; they can then show particular populations, change colours and scales etc. Note that there are also options to import worksheets from other experiments, and to save worksheets as pdfs. The default worksheet is where raw data is displayed but they can make a new raw worksheet if required. The default raw worksheet should not be overwritten. It is automatically linked with reference controls.

- Explain how they can check how well their unmixing has worked and show them how to add extra compensation if they should need it.

  Adding compensation: sometimes when unmixing doesn't work perfectly data can be adjusted by adding manual compensation. To do this they need to right-click on one sample then select Properties then Spillover. They should then tick the Enable Compensation box. To carry out the compensation they need to click on the Matrix icon in the worksheet toolbar. They can then either drag populations around or manually enter numbers in the matrix. Note that really they should check medians to do this properly. Right click on the tube to select Copy Spillover and then apply this to all the other samples by right clicking on the other samples and selecting Paste Spillover.

  The best way to check the unmixing is to look at the fully stained sample in plots for each parameter against one parameter (NxN plots). For example, keep BV605 on the x axis and change the y axis to look at how well each parameter has unmixed against BV605. Repeat this for each parameter. Mention that should they want to perform standard compensation on their data they can use Virtual Filters to do this (found in the Extra Tools module). A channel for each colour can be picked and the bandwidth increased if wanted. Compensated files can then be exported out along with the spillover matrix.

- Explain how the data is saved and exported. For very tube/well recorded two FCS files are saved; one raw, and one unmixed.

  Raw files contain information from every detector and these will be labelled by channel name (e.g. V1, V2), unmixed data contains data from only the channels in the experiment and will be labelled with the labels they have set up in their experiment (fluorochrome and target). To export data they should first make sure that their experiment and any settings they wish to save have been saved. Note that an asterisk after the experiment name, assay settings, or worksheet indicates that there is unsaved data there. They should always use the Save As function to prevent data being accidently overwritten – this is particularly important for any changes they may have made to the assay settings. To export their data they should close the experiment and then from the list of My Experiments they can select their experiment and then click on the Export button. This will generate a folder with two folders inside, one for raw data and one for unmixed data.

This folder should be saved in the D drive in Aurora Data Export. They should then copy this over to facsscratch or NextCloud. Tell users that if they need to make any changes to an experiment or import FCS files they should duplicate the experiment first – it is very easy to accidently overwrite data otherwise. This is also useful if they want to run unmixing with and without auto-fluorescence subtraction. They can duplicate the experiment with or without data.

- Go through cleaning the machine with them.

  Explain that they should use the Fluidics Shutdown command to clean the machine after their run. They should run this twice, the first time with one tube of 30% Contrad and then one tube of water. The second Fluidics Shutdown should be run with one tube of 10% bleach followed by one tube of water. Extra SIT flushes should be used between and after running their samples if sticky dyes such as PI are used. This is to help reduce carryover. If carryover is still seen or there is a clog they should run bleach on the SIT at a high flow rate followed by water for at least 5 minutes each. To shut-down the machine they need to run the Fluidics Shutdown twice and then turn off the cytometer and computer.

- Go through how to use the AMS if they will be regularly running plates (this can be done in a separate session).

  - Go through how to change from tubes to plates.

    They should ensure SIT is retracted before changing to plates. To turn on the loader use the button at the back of the loader. They should first remove the tube from SIT then pull the lever towards the front to bring the mixer forward. They should then go in to the Acquisition module and Acquisition Preferences to change the sample delivery mode from tubes to plates.

    Calibrate the SIT before running the plate. Note that only admin can change the position of this if it is wrong. This checks the SIT depth and the position of well A1. The SIT lift distance can be changed and is the distance in mm from the bottom of the plate.

  - Go through how to set up an experiment with a plate layout.

    In New Experiment they can select Add Plate. When they set up and experiment using a plate the plate display (Loader Settings) shows up. They can use drag and drop to order samples to match the order in the plate. They should assign wells as reference controls (R), samples (S), or cleaning (C) wells.

  - Go through the acquisition controls for plates and some key considerations for plate set-up.

    In the Acquisition Controls they can click on Eject to bring the stage forward. They can then load the pate (well A1 in front left corner). They should click on load and then start to begin acquisition. Note that the dead volume is anywhere between 70µl and 75µl. Cytek recommend using a minimum volume of 150µl per well. Note that they should only use low or medium flow rates when using the AMS as the high flow rate is really high(

100µl/min compared to 60µl/min in tubes). This can result in blockages and strange scatter patterns as the SIT becomes too close to the bottom and causes an air bubble/clog. Note that this can be changed by moving the SIT lift distance to 1-1.5 but obviously this increases dead volume. They can set the stage temperature, mix speed, time, and add options such as sample recovery here. They should note that most of these add time to each well. They can also set whether the wells are acquired row by row or column by column. Note that running in High throughput mode omits the wash and SIT flushes between wells.

- Go through cleaning the AMS.

  For cleaning the AMS they should add 2 wells of bleach and 2 wells of water to the end of the plate with a long mix setting to allow the probe to be cleaned. With sticky samples or when running a lot of wells in a plate they should add two wells of bleach and one of water at the end of each row in addition to the cleaning wells at the end of the plate. Extra SIT flushes should be used if sticky dyes such as PI are used to reduce carryover. If carryover is still seen or there is a clog they should run bleach on the SIT at a high flow rate followed by water for at least 5 minutes each.